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GENETIC AND PHYSIOLOGICAL CONTROL OF PROTECTIVE ANTIGEN SYNTHESIS BY BACILLUS ANTHRACIS

ANNUAL PROGRESS REPORT

CURTIS B. THORNE

DECEMBER 1980

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

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Amherst, Massachusetts 01003

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The primary objective of the research is to improve the yields of protective antigen in culture filtrates of Bacillus anthracis. Genetic and physiological factors controlling the synthesis and accumulation of protective antigen as well as the two other components of toxin are being investigated. The following areas are being pursued: (1) Isolation of mutants altered with respect to amounts of protective antigen and toxin produced; (2) Do toxin-producing strains carry plasmids and/or prophages that might be related to toxin synthesis; (3) Mapping of chromosomal genes; and (4) Assessment of physiological and

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20. Abstract (continued)

metabolic factors affecting protective antigen and toxin synthesis and accumulation.

Results of experiments to determine whether plasmid DNA is present in lysates of the Weybridge strain are ambiguous. It seems possible that a high molecular weight plasmid may be present. Experiments on induction of the Weybridge strain with ultraviolet light and mitomycin C suggest that the strain is a defective lysogen.

Several new phages active on the Weybridge strain have been isolated and they are being tested for transducing activity.

The Weybridge strain undergoes spontaneous variation (dissociation) at frequencies apparently much higher than one would expect for ordinary point mutations. Variants have been found that produce less protective antigen than the majority type but thus far none has been found that consistently produces more.

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Summary

This is a progress report (annual report) of research being carried out with Bacillus anthracis. The primary objective is to improve the yields of protective antigen in culture filtrates of B. anthracis. Genetic and physiological factors controlling the synthesis and accumulation of protective antigen as well as the two other components of toxin are being investigated. The following areas are being pursued: (1) Isolation of mutants altered with respect to amounts of protective antigen and toxin produced; (2) Do toxin-producing strains carry plasmids and/or prophages that might be related to toxin synthesis; (3) Mapping of chromosomal genes involved in toxin synthesis if such genes are not carried by a plasmid or prophage; and (4) Assessment of physiological and metabolic factors affecting protective antigen and toxin synthesis and accumulation. Although the contract for the research has been in effect only six months, some progress has been made in most of the designated areas.

Results of experiments to determine whether plasmid DNA is present in lysates of the Weybridge (Sterne) strain are ambiguous. However, the most recent experiments suggest that a plasmid of fairly high molecular weight may be present. Experiments on induction of the Weybridge strain with ultraviolet light and mitomycin C indicate that the strain is lysogenic. To date no strains of a number of Bacillus thuringiensis and Bacillus cereus tested appear to be sensitive to the presumed phage. This raises the possibility that the phage in question may be defective.

Phage CP-51 is effective in mediating transduction in the Weybridge strain but the virulent nature of CP-51 causes problems in recovery of transductants and in their analysis for linkage. Therefore, efforts are being made to find a more temperate transducing phage. Attempts to find host-range mutants of temperate transducing phages for B. thuringiensis that are active on B. anthracis have been unsuccessful. However, several new phages active on the Weybridge strain have been isolated from soil and other sources and these are being screened for the ability to transduce.

The Weybridge strain undergoes spontaneous variation at frequencies apparently much higher than one would expect for ordinary point mutations. Spontaneous variants have been isolated that differ from the majority type in (1) colonial morphology, (2) frequency of sporulation, (3)nutritional requirements, (4) sensitivity to bacteriophages, and (5) amounts of protective antigen produced. Variants have been found that produce less protective antigen than the majority type, but thus far none has been found that consistently produces more.

Foreward

Citations of trade names in this report does not constitute an official Department of Army endorsement or approval of the use of such items.

TABLE OF CONTENTS

Although this report is formally designated as an annual report, because it is being submitted in conjunction with application for renewal of the contract, it, in fact, represents only six months of work (July 1, 1980 to December 31, 1980). The research proposal submitted for the contract proposed the following areas of investigation: (I) Isolation of mutants altered with respect to amounts of protective antigen and toxin produced with the aim of finding a hyperproducing mutant; (II) Do toxin-producing strains carry plasmids, and if so, is the presence of one or more plasmids related to toxin synthesis; (III) Do toxin-producing strains carry prophage, and if so, is the presence of prophage related to toxin synthesis; (IV) Mapping of chromosomal genes involved in toxin synthesis; and (V) Assessment of physiological and metabolic factors affecting protective antigen and toxin synthesis and accumulation. During the past six months we have begun investigation of all the areas mentioned and have made some progress in each of them. In the following report each of the areas is discussed individually following a general description of materials and methods. Specific procedures which themselves are results of the research are described as appropriate under individual sections.

MATERIALS AND METHODS

Organisms. The following avirulent strains of Bacillus anthracis and derivatives of them have thus far been included in our study: Weybridge (Sterne) (1); Anvax, obtained from Jensen-Salsbery Laboratories, Kansas City, MO; V770, obtained from Ms. Anna Johnson, USAMRIID, Fort Detrick, MD; and CN 18-74 (Sterne) (2) obtained from G. Ivanovics, Szeged, Hungary.

Media and buffers. For convenience to the reader and for ease of future reference, compositions of the various media and buffers used are given in detail. All amounts are for one liter, final volume. For preparation of solid growth media 15 g of Difco agar were added per liter of the corresponding broth.

NBY broth: Difco nutrient broth, 8 g; Difco yeast extract, 3 g.

L-broth: Difco tryptone, 10 g; Difco yeast extract, 5 g; NaCl, 10 g. pH adjusted to 7.0 with NaOH.

PA (phage assay) broth: Difco nutrient broth, 8 g; NaCl, 5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4 \cdot H_2O$, 0.05 g; $CaCl_2 \cdot 2H_2O$, 0.15 g; pH adjusted to 6.0 with HCl.

Minimal I: $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 6 g; K_2HPO_4, 14 g; sodium citrate, 1 g; glucose, 5 g; L-glutamic acid, 2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeCl_3 \cdot 6H_2O$, 0.04 g; $MnSO_4 \cdot H_2O$, 0.00025 g; pH adjusted to 7.0 with NaOH. The glucose and $FeCl_3$ were sterilized separately.

Minimal IB: To Minimal I was added 10 mg of thiamine hydrochloride and 160 mg of each of the following amino acids: L-methionine,

L-leucine, L-valine, L-alanine, L-serine, L-threonine, L-proline, L-phenylalanine.

Minimal 3: To Minimal I was added 10 mg of thiamine hydrochloride and 200 mg of glycine.

Minimal 4NH: To Minimal I was added 10 mg of thiamine hydrochloride, 200 mg of glycine, 40 mg of L-methionine, 40 mg of L-histidine, and 10 mg of nicotinamide.

Minimal H: To Minimal I was added 10 mg of thiamine hydrochloride, 200 mg of glycine, and 40 mg of each of the following amino acids: L-methionine, L-alanine, L-serine, L-threonine, L-proline, and L-phenylalanine.

Minimal M: To Minimal I was added 10 mg of thiamine hydrochloride, 200 mg of glycine, and 40 mg of L-methionine and L-proline.

Minimal O: To Minimal I was added 10 mg of thiamine hydrochloride, 200 mg of glycine, and 40 mg of L-methionine, L-serine, L-threonine, and L-proline.

Lysis medium for B. anthracis: Combine 10 ml of double strength (2X) Difco Brain Heart Infusion broth, 15 ml of 2X Minimal I broth (glucose and $FeCl_3$ omitted) and 25 ml of 40% sucrose.

Agar for immunodiffusion: KH_2PO_4, 10 g; NaCl, 9 g; agarose, 7.5 g; pH adjusted to 7.3 with NaOH. After autoclaving 0.1 g of thimerosal was added.

Gelatin-phosphate diluent: Na_2HPO_4, 2.8 g; Difco gelatin, 5 g; pH adjusted to 8.0.

Tris acetate buffer: Tris base, 6.05 g; sodium acetate, 2.72 g; NaCl, 1.05 g; ethylenediamine tetra-acetic acid, 0.716 g; pH adjusted to 8.0 with acetic acid.

TES buffer: Tris base, 6.05 g; NaCl, 2.9 g; ethylenediamine tetra-acetic acid, 1.6 g; pH adjusted to desired value.

Antiserum: The antiserum was supplied by Ms. Anna Johnson of USAMRIID, Fort Detrick, and is identified as Horse RB, 26 Nov. 1980. It was prepared in a horse by injecting spores of Sterne strain.

Immunodiffusion method of antigen assay. The procedure was modified from that of Thorne and Belton (1). The composition of the diffusion agar is given under Media and buffers. Samples were diluted in gelatin-phosphate. The array of antigen and antibody wells was that of a template obtained from Miles Laboratories, Inc. A center well 4 mm in diameter is surrounded by six wells 5 mm in diameter and spaced 3 mm from the center well. With the current batch of antiserum (Horse RB, 26 Nov. 1980) the sensitivity with this array appears to be similar to that obtained with the template used by Thorne and Belton (1) which had larger wells farther apart. However, the assays can be read sooner with gels made from the smaller template. The longer time required for the lines to develop on plates with the larger pattern of wells suggests that the antiserum currently in use is not as active as the antisera used in the earlier studies (1, 3, 4).

RESULTS AND DISCUSSION

I. Isolation of mutantd altered with respect to amounts of protective antigen and toxin produced.

Observations in my laboratory during the past three or four years (Thorne, C.B., unpublished) have shown that strains of Bacillus thuringiensis undergo spontaneous variation (sometimes referred to as dissociation) with respect to colonial morphology, nutritional requirements, ability to sporulate, ability to produce the parasporal crystal or toxin, and susceptibility to bacteriophages apparently at frequencies much higher than one would predict for simple point mutations. Now in our studies with B. anthracis, we find that the Weybridge strain and other Sterne-type strains are similar in this respect to B. thuringiensis. We have observed high frequency variation with respect to colonial morphology, frequency of sporulation, nutritional requirements, sensitivity to bacteriophage, and yields of protective antigen.

Populations grown from single wild-type colonies usually exhibited various types of colonial morphology when cells were plated on L-agar or NBY agar. Usually two types predominated but more rare types were also seen. To be certain that the colony types represented true variants rather than contaminants, auxotrophic mutants were included in the studies on variation and the variants were tested for retention of the auxotrophic marker. Some of the variants were tested for antigen production and results are summarized in Table 1. For example, M44 represents a tryptophan auxotroph of the Weybridge strain and M44A and M44B are colonial variants isolated from M44. Anvax A, B, and C of Table 1 represent three types of colonial variants found when spores of the Anvax strain were streaked on NBY agar.

Variation with respect to nutritional requirements of the Weybridge strain is also being investigated. In studies on growth of B. anthracis in synthetic media several years ago, I found that Minimal IB, which is simpler than the synthetic medium formulated by Brewer, et al. (5), would support good growth of several strains (Thorne, C.B., unpublished). In the current studies I found that one variant of Weybridge (Variant A, Table 1) grew very well on this medium but that another variant of Weybridge (Variant B, Table 1) grew very poorly. When cells of variant B were streaked on agar plates of Minimal IB, most cells gave only very poor growth (inadequate) but

a few cells grew into well-developed colonies similar to those produced by variant A; presumably they were new type A variants arising in the population. Further tests showed that cells of Weybridge variant A were capable of growing well and producing large colonies on media considerably simpler than Minimal IB. For example, Minimal H gave better growth than Minimal IB; Minimal O was as good as Minimal N and Minimal M gave adequate growth.

When mutant M44 (Trp^-) was streaked on Minimal 4NH (supplemented with tryptophan) occasional cells produced well-developed colonies. These could be transferred time after time on Minimal 4NH and good growth always resulted. An example of a variant of M44 that grew well on 4NH is M44-1 of Table 1. In addition to having simpler nutritional requirements, it also sporulated very rarely and as Table 1 shows, it did not produce any detectable protective antigen under our standard conditions. It should be pointed out that M44-1 retained the Trp^- marker of the parent strain.

This phenomenon of spontaneous variation at high frequencies is proving to be one of the most perplexing problems we have encountered in our work on B. anthracis and it is probably one of the most important. For consistent and dependable production of protective antigen it would be desirable to have a stable strain. The reason for the instability is not known but we plan to investigate this. As Table 1 shows we have found variants that produce less protective antigen than the parent strain, but thus far we have found none that produces more. It seems reasonable that hyperproducers may also occur and we will continue looking for them among spontaneous variants as well as among mutagenized cultures.

II. Do toxin-producing strains carry plasmids?

Methods available for lysis of cells of Bacillus species for the purpose of chromosomal and/or plasmid DNA isolation were not applicable to B. anthracis. Several methods were tried without success. However, we have been successful in devising a procedure which is very effective with the Weybridge strain and it is outlined below.

Lysis of cells for DNA isolation. Spores (approximately 10^7 in 0.1 ml) were inoculated into 50 ml of lysis medium in a 250-ml Erlenmeyer flask which was then incubated on a shaker at 37°C. After the optical density (O.D.) at 660 nm reached 0.45, one ml of D-cycloserine (50 mg/ml) was added and incubation was continued until the O.D. reached 0.9. The cells were collected by centrifuging 40 ml per tube in a Sorvall centrifuge (SS-34 rotor) at 12,000 rpm for 15 min at 5°C. Each pellet of cells was suspended in 2 ml of 2X TES buffer at pH 10 (this yielded a suspension having a pH of 8.5). One ml of lysozyme (5 mg/ml in TES buffer, pH 8.5) was added and the suspension was incubated statically in a 37°C water bath for 30 min with occasional mixing. One ml of pronase (5 mg/ml) was added, followed by the addition of SDS to give a final concentration of 0.7%, and the suspension was held in a 65°C water bath for 30 min with occasional mixing. Sarkosyl was finally added to give a final concentration of 2% and the suspension was incubated for an additional 15 min at 65°C. This procedure produced clear lysates consistently.

Separation of plasmid DNA from chromosomal DNA. To look for plasmid DNA we subjected the lysate to the alkali denaturation procedure described by Currier and Nester (6). The dialyzed DNA was then centrifuged in CsCl-ethidium bromide in a SW 50.1 rotor on a Beckman Model L centrifuge at 34,000 rpm for 48 hours. The denser band, presumed to be plasmid DNA, and the less dense chromosomal DNA band were removed separately and dialyzed against two successive liters of TES buffer at pH 8.0 for a total of 24 hours at 4°C.

Gel electrophoresis. Horizontal slab gels contained 0.6% low EEO agarose (Type I, Sigma Chemical Co.) in Tris acetate buffer, pH 8.0. Best results were obtained by loading 45 µl of a mixture containing 10 µl of bromthymol blue (50 µg/ml), 10 µl of 1% agarose, and 0.1 ml of DNA solution into the well. The wells were sealed with a drop of 1% agarose and the slab was covered with 10 ml of Tris acetate buffer and Saran Wrap. The gels were run at 35 milliamperes for 12 to 14 hours. They were stained for 30 min in Tris acetate buffer containing 0.1 µg of ethidium bromide per ml and then destained in distilled water for 15 to 30 min.

Search for plasmid DNA in cell lysates. Results of experiments in which we looked for plasmids in DNA from the Weybridge strain are ambiguous. Results of early experiments suggested that at least one plasmid was present but this has not been consistently reproducible. In our most recent experiments, however, more evidence for a plasmid has been obtained. I tend to believe that a plasmid of rather high molecular weight is present and its high molecular weight probably accounts for our failure to detect it consistently. It is not uncommon for investigators to fail to detect the presence of plasmids having high molecular weights because of their poor separation from chromosomal DNA by gel electrophoresis. We hope to resolve this problem soon by starting with larger amounts of DNA and trying to refine the procedure for removing chromosomal DNA before electrophoresis.

Test for plasmids carrying antibiotic resistance. Before we began to look for plasmid DNA in cell lysates, we tested the Weybridge, Anvax, and V770 strains for resistance to a number of antibiotics with the idea that plasmids conferring drug or antibiotic resistance might be present. For instance, when we did this with B. thuringiensis strains in our collection we found one that was resistant to tetracycline, and the resistance determinant was carried on a plasmid. However, all three strains of B. anthracis tested were normally sensitive to chloramphenicol, nalidixic acid, streptomycin, oxacillin, spectinomycin, kanamycin, tetracycline, neomycin, novobiocin, actinomycin D, bacitracin, and tyrothricin.

III. Do toxin-producing strains carry prophage?

Attempts to demonstrate lysogeny in the Weybridge strain by cross-streaking cells against a large number of B. thuringiensis and B. cereus strains were unsuccessful in that no evidence of lysis or inhibition of growth of any of the test strains was seen. However, experiments on induction of the Weybridge strain with ultraviolet light or mitomycin C strongly suggest that the strain is lysogenic.

For induction with ultraviolet light 0.1 ml of an overnight culture of the Weybridge strain in L-broth was transferred to 6 ml of L-broth in a 20-mm tube and incubated on a shaker at 37°C for 5 hours. Two ml were transferred to a petri dish and exposed to UV light for 30 seconds (two General Electric 15 watt germicidal bulbs at a distance of 16 inches from the petri dish). The exposed cells were transferred to 6 ml of L-broth in a 20-mm tube and incubated on the shaker at 37°C overnight (about 14 hours). Lysis of the cells was evident.

For induction with mitomycin C two ml of a 5-hour L-broth culture prepared as above were transferred to 6 ml of L-broth in a 20-mm tube. Mitomycin C was added to give a final concentration of 0.15 µg/ml and the culture was incubated on the shaker at 37°C for 14 hours at which time lysis was apparent.

The above lysates were tested against 17 strains of B. thuringiensis, 6 strains of B. cereus, and one strain of B. brevis by streaking cells of the cultures across an area on an agar plate which had been spread with a lysate. Both L-agar and PA agar were used in these tests since previous experience with phages of B. thuringiensis and B. cereus had shown that the results of lysis tests can vary with the test medium. None of the test strains appeared to be lysed or inhibited by either lysate.

Although we will continue to look for a positive indicator strain, these results suggest that the Weybridge strain may be carrving a defective prophage. We hope to resolve this question soon by examining concentrated lysates under the electron microscope to see whether phage-like particles are present. Defective phages are common among Bacillus species and it is not unlikely that the Weybridge strain carries one or more. Nagy and Ivanovics (2) reported that two Sterne strains, CN 18-74 and CN 35-18, are defective lysogens, but no attempt was made by those investigators to relate the presence of prophage to production of toxin or protective antigen. Our results on protective antigen production summarized in Table 1 indicate that strain CN 18-74 did not produce any detectable protective antigen under the conditions of our test. We plan to investigate this further.

IV. Mapping of chromosomal genes involved in toxin synthesis.

If toxin genes are on the chromosome rather than on a plasmid or prophage, it will be desirable to locate them with respect to other markers by genetic mapping techniques. Since there have been no genetics studies done with B. anthracis there is no background of knowledge to draw upon, and it is obvious, therefore, that considerable preliminary work has to be done before toxin genes can be mapped.

Although phage CP-51, which was isolated in my laboratory and used in transduction studies with B. cereus (7) and B. thuringiensis (8), is also capable of mediating transduction in B. anthracis (9), its virulent nature creates problems in recovering and analyzing transductants quantitatively. Thus, it would be very desirable to have a more temperate phage to use in chromosomal mapping of B. anthracis. We have tested our other transducing phages for B. thuringiensis, i.e., TP-10, TP-11, TP-12, TP-13, and TP-18, which are quite temperate and useful for chromosomal mapping in that species, for activity on the Weybridge strain and all were inactive. We have made a few unsuccessful attempts to find host-range mutants that would be active on B. anthracis and we will pursue this attack further. In the meantime, we

have isolated from soil several new phages that are active on the Weybridge strain and we are now in the process of screening them for transducing activity.

We have developed an effective method for isolating auxotrophic mutants of B. anthracis. The Iyer (10) technique for isolating mutants following UV treatment of wild-type spores has been used very successfully in my laboratory for several years for isolating mutants of B. subtilis and B. licheniformis. However, we experienced difficulty in applying the method to B. anthracis, just as we had previously experienced with B. cereus and B. thuringiensis. The problem appears to be in the propensity of these organisms to grow in long chains.

The following method has been used successfully with all three of the above species. A sample of culture grown in L-broth or NBY broth is treated with UV light and transferred to fresh medium and allowed to incubate at 37°C for several hours. A sample from that culture is then treated with UV light as before and transferred to fresh medium and allowed to grow for several hours. This procedure is repeated for a total of four or five times to allow mutants to accumulate in the population. Finally samples of the mutagenized culture are transferred to a good sporulation medium, e.g., NBY broth or agar for B. anthracis, and allowed to sporulate so that mutants will segregate into single colony-forming units. Appropriate dilutions of the sporulated culture are plated on enriched minimal agar and after 24 to 48 hours at 37°C the tiniest colonies are picked and tested for auxotrophy.

We are in the process of collecting and characterizing auxotrophic mutants of the Weybridge strain which will be useful in mapping studies. In addition, we plan to screen cultures mutagenized in this manner for hyper-production of protective antigen.

V. Assessment of physiological and metabolic factors affecting protective antigen synthesis and accumulation.

In December 1980 Ms. Anna Johnson of USAMRIID at Fort Detrick provided me with antiserum from a horse injected with spores of avirulent B. anthracis, and thus we are now able to determine the amounts of protective antigen in culture filtrates by immunodiffusion. Consequently, we have begun studies on physiological and metabolic factors affecting yields of protective antigen.

In our first tests we learned that protective antigen is adsorbed to Millipore membranes when small volumes of culture fluid are sterilized by filtration. We are able to prevent such adsorption by adding a small amount of serum or gelatin to samples before filtering an antigen sample. Our standard procedure of growing 100 ml of culture in 250-ml flasks and quantitating the protective antigen in culture filtrates by immunodiffusion will lend itself to routine testing of a large number of mutants as well as variations in growth medium and other cultural conditions.

Some of our results on protective antigen production are summarized in Table 1. Our results confirm those of Thorne and Belton (1) in that higher yields were obtained in the hydrolyzed casein medium than in completely synthetic media. Thus far we have not obtained antigen titers as high as those reported by Thorne and Belton (1). Some possible reasons under consideration are: (a) strain variation, (b) failure to reproduce the medium exactly as used by Thorne and Belton, (c) the titers may appear lower than they really are because of differences in the antisera. Nevertheless, we can reproduce the titers consistently and therefore we have a good working system.

Table 1. Yields of protective antigen from various strains of B. anthracis

Strain	Medium	Units of protective antigen/ml[1]
Weybridge (wild type)	Casamino acids[2]	4
Weybridge Variant A	"	4
Weybridge Variant B	"	4
Weybridge M44	"	4
Weybridge M44A	"	2
Weybridge M44B	"	8
Weybridge M44-1	"	<1
Anvax (wild type)	"	4
Anvax Variant A	"	2
Anvax Variant B	"	2
Anvax Variant C	"	4
V770	"	4
CN 18-74	"	<1
Weybridge (wild type)	1095[3]	<1
Anvax (wild type)	"	±1
V770	"	±1

[1]The number of units is the reciprocal of the highest dilution showing a line of precipitate. Serial two-fold dilutions were tested.

[2]The Casamino acids medium of Thorne and Belton (1).

[3]The synthetic medium number 1095 of Wright et al. (11).

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